

ALKALOID CONTENT OF SOME POTENTIAL ISOFLAVONOIDS SOURCES (NATIVE *GENISTA* SPECIES). LONG-TERM SAFETY IMPLICATIONS

AMELIA TERO-VESCAN¹, CAMIL-EUGEN VARI^{2*}, LAURIAN VLASE³

¹University of Medicine and Pharmacy Tîrgu Mureş, Faculty of Pharmacy, Department of Pharmaceutical Biochemistry, 38th Gheorghe Marinescu Street, Tîrgu Mureş, Mureş, 540139

²University of Medicine and Pharmacy Tîrgu Mureş, Faculty of Pharmacy, Department of Pharmacology and Clinical Pharmacy, 38th Gheorghe Marinescu Street, Tîrgu Mureş, Mureş, 540139

³University of Medicine and Pharmacy "Iuliu Hațieganu", Faculty of Pharmacy, Department of Pharmaceutical Technology and Biopharmaceutics, 13th, Emil Isac Street, Cluj-Napoca, Cluj 400023

*corresponding author: camil.vari@umftgm.ro

Abstract

The purpose of this study was to determine the quinolizidine alkaloids content – cytosine and sparteine – of some *Genista* extracts, using both extraction methods specific for alkaloids and for isoflavonoids. *Genista* species are known to be sources of isoflavonoids, but the risk of concomitant extraction of alkaloids was not assessed. A LC-MS/MS method with electrospray ionisation was used and cytosine levels between 0.093 to 6.588 mg/100 g dry plant were obtained, while sparteine was present only in trace amounts. This content is not toxic to humans at an isoflavonoids content recommended for daily intake, but is close to therapeutic levels (use to treat tobacco addiction). As a result of this study, unpurified extracts of native species of *Genista* are not recommended for phytoestrogenic effects.

Rezumat

Scopul acestui studiu a fost determinarea conținutului în alcaloizi chinolizidinici – citizina și sparteina al unor extracte din specii de *Genista* utilizând metode de extracție atât specifice pentru alcaloizi, cât și pentru izoflavonoide. Speciile de *Genista* sunt cunoscute ca fiind surse de izoflavonoide, dar nu a fost evaluat riscul extracției concomitente a alcaloizilor. S-a utilizat o metodă LC-MS/MS cu *electrospray ionisation* și s-au obținut nivele de citizina între 0,093-6,588 mg/100 g plantă uscată, iar sparteina a fost prezentă doar în cantități nesemnificative. Acest conținut este netoxic pentru om conform studiilor de inocuitate la un conținut de izoflavonoide recomandat ca aport zilnic, dar este apropiat de nivele la care apar efecte terapeutice (utilizare pentru tratamentul adicției la tutun). Ca urmare a acestor rezultate, nu recomandăm utilizarea extractelor nepurificate a speciilor indigene de *Genista* pentru acțiunea fitoestrogenică.

Keywords: cytosine, sparteine, isoflavonoids, LC-MS/MS method

Introduction

Genista tinctoria L and *Genista sagittalis L* are plants from the *Fabaceae* family, very well-spread in our country, whose potential use due to the isoflavonoids content is limited by the presence of quinolizidine alkaloids such as cytisine or sparteine. There are numerous studies on the phytoestrogenic effects of isoflavonoids like genistein or daidzein in breast or prostate cancer chemoprevention, in preventing osteoporosis, postmenopausal symptoms [13], in preventing obesity and type II diabetes [1]. Cytisine is a quinolizidine alkaloid acting as a partial agonist on nicotinic receptors in the brain and particularly the $\alpha 4\beta 2$ subtype. Because of these effects cytisine was used for smoking cessation [6]. Sparteine presents antimuscarinic effects [5], oxytocic and also toxic effects – cardiac arrhythmia, neurological disorders (mydriasis, dizziness), gastrointestinal disorders [7].

Plant extracts are commonly consumed in phytotherapy, products classified as dietary supplements that are not controlled in terms of highly active, potentially toxic compounds, either due to lack of literature data or because of permissive laws [14].

Nowadays, there are numerous studies on the phytoestrogen content of different species from *Fabaceae* family [2, 8] therefore the purpose of this study was to determine the cytisine and sparteine content in different extracts of the two plants from the *Fabaceae* family - *Genista tinctoria L* and *Genista sagittalis L* by a LC-MS/MS method and to assess the potential effects these compounds have, in the quantity found in plants, considering the isoflavonoids content and the possibility to administrate them as phytoestrogens.

Materials and Methods

Chemicals, reagents, solvents

Standards: Sparteine and cytisine were purchased from Fluka (Steinheim, Germany) and are of high purity > 98%. Methanol, ammonium acetate and hydrochloric acid, reagents of analytical grade were bought from Merck KGaA (Darmstadt, Germany). Distilled water was obtained from a Millipore Direct Q system.

Preparation of standard solutions

Cytisine calibration curve was prepared in the concentration range of 11.2 to 1120 ng/mL and the sparteine calibration curve was prepared in the concentration range of 9.78 to 978 ng/mL. All solutions were prepared in distilled water.

Extraction method from plants

Genista tinctoria L and *Genista sagittalis L* were collected during the flowering period in the area near the city of Tîrgu Mureş, Romania. Samples were diluted 1:10 with distilled water before analysis. The following extracts were performed:

a. Alkaloids extraction with methanol by heating under reflux

The aerial parts of the plant were ground; 5 g of plant powder was heated under reflux for 4 hours with 100 mL of absolute methanol. The solution was filtered and the total volume was adjusted to 100 mL by washing the filter with methanol.

b. Alkaloids extraction with 75⁰ methanol by heating under reflux

The extraction method is the same as that described in point *a*, but using 75⁰ methanol as an extraction solvent.

c. Alkaloids extraction with methanol in ultrasonic bath

The aerial parts of the plant were ground; 5 g of plant powder was stirred in an ultrasonic bath for 4 hours with absolute methanol. The solution was filtered and the total volume was adjusted to 100 mL by washing the filter with methanol.

d. Alkaloids extraction with 75⁰ methanol in ultrasonic bath

The extraction method is the same as that described in point *c*, but using 75⁰ methanol as an extraction solvent.

e. Alkaloids extraction with 0.05 N HCl

The aerial parts of the plant were ground, 5 g of plant powder was macerated for 24 hours, in the dark, with 100 mL HCl 0.05 N. After 24 hours, the extract was left in the ultrasonic bath for two hours. The solution was filtered and the total volume was adjusted to 100 mL with 0.05 N HCl.

f. Alkaloids extraction with a mixture 0.05 N HCl: methanol (3:1)

The extraction method is the same as that described in point *e* but using for extraction a mixture of 0.05 N HCl: methanol (3:1).

Table I.
Coding of vegetal samples extracts

Species	Extraction pathway	Solvent	Coding
<i>Genista tinctoria L</i>	reflux extraction	absolute methanol	GTRM
		75 ⁰ methanol	GTRM75
	ultrasound bath	absolute methanol	GTUM
		75 ⁰ methanol	GTU75
		HCl 0.05 N	GTH
		HCl 0.05 N: methanol (3:1)	GTHM
<i>Genista sagittalis L</i>	reflux extraction	absolute methanol	GSRM
		75 ⁰ methanol	GSRM75
	ultrasound bath	absolute methanol	GSUM
		75 ⁰ methanol	GSU75
		HCl 0.05 N	GSH
		HCl 0.05 N: methanol (3:1)	GSHM

Chromatographic conditions

HPLC system equipped with mass spectrometer consisting of: HP 1100 Series binary pump, autosampler HP 1100 Series, HP 1100 Series thermostat, Agilent Ion Trap 1100 SL mass spectrometer.

Due to the different chemical characteristics of quinolizidine alkaloids present in plant extracts, two methods were developed: one to determine sparteine and one for determining cytisine. Separation was performed on a Gemini-C18 50 mm x 3.0 mm i.d., 3 μ m (Phenomenex, SUA) analytical column. The mobile phase used for cytisine determination was a mixture of ammonium acetate 1 mM with 0.1% ammonia, (pH=10) and methanol 82/18 (v/v) at a flow rate of 1 mL/min and at a temperature of 32^o C. In the case of sparteine a mixture of 1 mM ammonium acetate with 0.1% ammonia (pH=10) and acetonitrile 40/60 (v/v) was used at a flow rate of 1 mL/min and a temperature of 32^o C.

MS conditions:

- ion source: ESI (electrospray ionisation);
- ionization mode: positive;
- nebulizer nitrogen, 65 psi pressure;
- drying gas: nitrogen, flow rate 12 L/min, temperature 360 C;
- capillary potential: 2000 V;
- analysis mode: monitoring transitions m/z 191>148.

Results and Discussion

Determination of cytisine content in plant extracts

MS/MS detection in case of cytisine was performed by fragmentation of ion with m/z 191, corresponding to protonated molecule of cytisine, and then monitoring the ion with m/z 148 from the MS/MS spectrum of the analyte. Injection volume was 1.5 μ L. Retention time: 1.4 minutes. Total analysis time: 2.2 minutes.

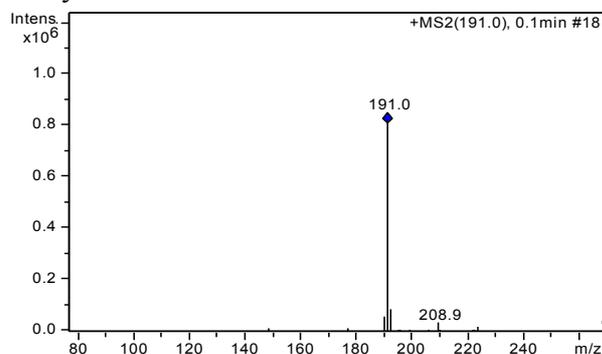


Figure 1.

Full-scan mass spectrum of cytisine in the mobile phase

To increase the selectivity of the HPLC/MS method, fragmentation was performed on the characteristic cytosine ion (m/z 191) and the MS/MS spectrum was recorded (Figure 2).

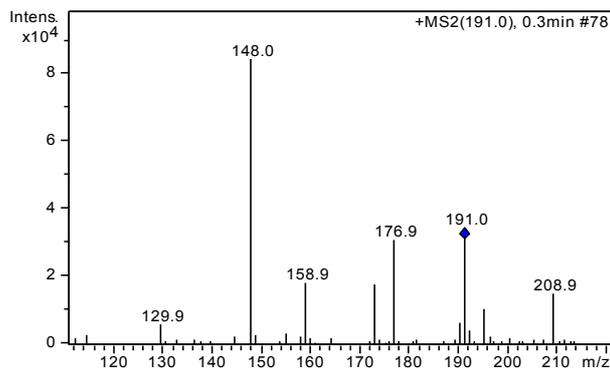


Figure 2.

The MS/MS spectrum of cytosine in the mobile phase

By fragmentation, cytosine can be split into several fragments, of which the main one, which was chosen for quantification has m/z 148.

In Figure 3 the chromatogram of a cytosine standard sample (concentration 11.2 ng/mL) after MS detection is presented.

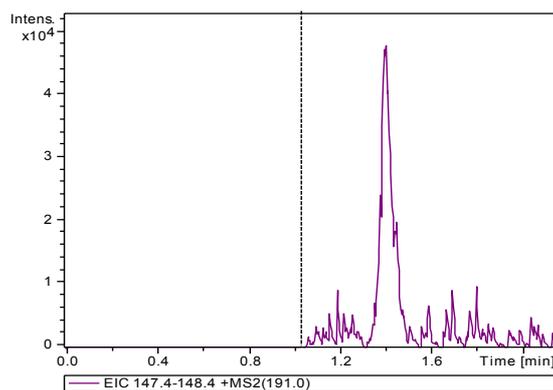


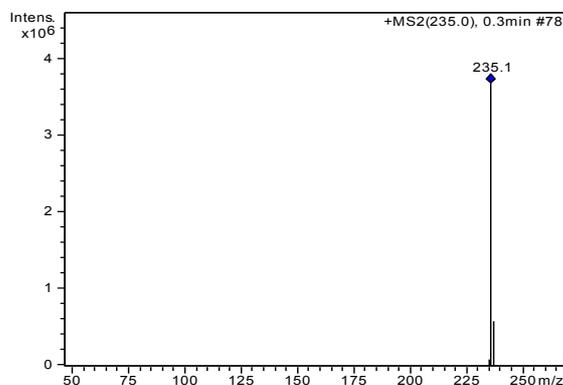
Figure 3.

Chromatogram of cytosine by MS/MS detection (concentration 11.2 ng/mL)
RT=1.4 min

Cytosine concentration was determined based on area under the curve by an external standard method. The calibration curve for cytosine, by MS/MS detection, followed the quadratic model $y=ax^2+bx+c$, where y is the AUC, and x is the analyte concentration, was $y = -2.79x^2 + 8784 x - 9294$ with $R^2 > 0.9999$, $N = 6$ points, $n = 3$ determinations.

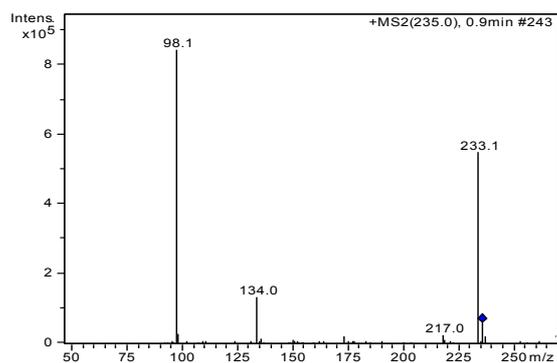
Determination of sparteine content in plant extracts

Mass spectrum (full-scan) of a sparteine solution recorded under the described conditions is shown in Figure 4. The expected ion in accordance with the monoisotopic molecular weight of sparteine ($M=234$) and by ionization mode (positive) is ion with m/z 235, corresponding to the protonated molecule.

**Figure 4.**

Full scan mass spectrum of sparteine in the mobile phase

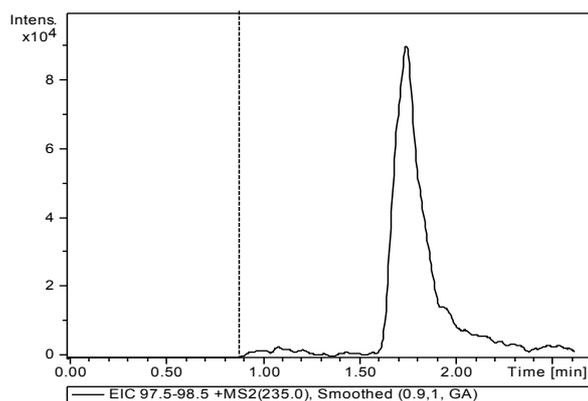
To increase the selectivity of the HPLC/MS method, fragmentation was performed on the characteristic sparteine ion (m/z 235) and the MS/MS spectrum was recorded (Figure 5).

**Figure 5.**

The MS/MS spectrum of sparteine in the mobile phase

By fragmentation, sparteine can be split into several fragments, of which the main one, which was chosen for quantification, has m/z 98.

In Figure 6 the chromatogram of a sparteine standard sample (concentration 9.78 ng/mL) after MS detection is presented.

**Figure 6.**

Chromatogram of sparteine by MS/MS detection (concentration 9.78 ng/mL), RT=1.72 min

Sparteine calibration curve (quadratic model) by MS/MS detection is: $y = -9.78x^2 + 48324x$ with a coefficient $R^2 > 0.999$, $N = 6$ points, $n = 3$ determinations.

Under these chromatographic conditions the obtained results are shown in Tables II and III.

Table II.

Cytisine content in *Genista tinctoria L* and *Genista sagittalis L* extracts

Species	<i>Genista tinctoria L</i>	Species	<i>Genista sagittalis L</i>
Extract	Cytisine mg/100 g dry plant	Extract	Cytisine mg/100 g dry plant
GTRM	0.543	GSRM	0.093
GTRM75	1.447	GSRM75	0.314
GTUM	0.322	GSUM	3.917
GTU75	0.792	GSU75	5.022
GTH	1.496	GSH	4.488
GTHM	2.350	GSHM	6.588

Table III.

Sparteine content in *Genista tinctoria L* and *Genista sagittalis L* extracts

Species	<i>Genista tinctoria L</i>	Species	<i>Genista sagittalis L</i>
Extract	Sparteine mg/100 g dry plant	Extract	Sparteine mg/100 g dry plant
GTRM	0.015	GSRM	0
GTRM75	0.010	GSRM75	0
GTUM	0.007	GSUM	0
GTU75	0.007	GSU75	0
GTH	0.002	GSH	0
GTHM	0.009	GSHM	0

Cytisine is present in the studied *Genista* species extracts, but its concentration may be considered safe. Considering the potential applications of cytisine in smoking cessation at a maximum recommended dose of 9 mg, using isoflavonoids extracts, presents no toxic risks because alkaloids are present in lower doses than those used in therapy. Although there are available studies concerning *in vivo* and *in vitro* hepatic toxicity of cytisine (modification in hepatocytes viability, decreased levels of reduced glutathione, increased values of serum transaminases and lactate dehydrogenase (as a marker of cell membrane integrity) that manifests in a dose dependent manner, it is less toxic than nicotine. Cytisine is not genotoxic, as cytogenetic tests for *in vivo* aberrations in the bone marrow cells of mice showed and extrapolation of these data to humans indicate no risk of clastogenic activity [12].

Experimental data show that sparteine is not present in *Genista sagittalis* L. Sparteine content in *Genista tinctoria* L is also very low (0.002-0.015 mg/100 g dry plant) and if we consider that the LD₅₀ in rats after oral administration is with several orders of magnitude greater, we can conclude that there is no risk of acute toxicity in humans. Sparteine metabolism in humans is CYP2D6-dependent and presents genetic polymorphism [10]. 7-10% of caucasians have a genetic polymorphism of CYP2D6 manifested by the absence of enzyme activity (poor metabolisers) [4, 9].

In case of cytisine, CYP450-dependent metabolism studies are insufficient to characterize the substance considering regulatory requirements.

Genista tinctoria L average isoflavonoids content in 100 g dry plant, expressed in genistein is approximately 50 mg (the recommended intake for postmenopausal women) [11], which also brings a cytisine content of about 1.15 mg/100g dried plant. Alkaloids extraction is usually performed by using acid medium, but the results show that solvents used for obtaining isoflavonoid-rich extracts, in this case, also yield appreciable alkaloid content. Following the use of unpurified isoflavonoid extract (without removing alkaloids) exposes to about 1/3 of the maintenance dose (3 mg/day in the last phase of therapy) used in some countries (Bulgaria, Poland) for smoking cessation [3]. Although that dose is without health risk, including for long-term use, we believe it would not be useful exposing the patient to a cumulative dose equivalent to the pharmacological dose, as there are other sources of isoflavonoids with a good extraction efficiency and with other economic applications (*Glycine max* L).

Conclusions

Extracts from *Genista* species have an important content of isoflavonoids, their extraction is accompanied by obtaining a low content of

alkaloids, without significant toxic risk, but close to the doses currently used in therapy, which may limit their use only for phytoestrogenic effects. If the source of phytoestrogens is represented by species of *Genista* (which contain quinolizidine alkaloids) extract purification is recommended, but this involves extra expenses which make them unprofitable considering the abundant alternative sources of phytoestrogens.

References

1. Behlou N., Wu G., Genistein, A promising therapeutic agent for obesity and diabetes treatment. *European Journal of Pharmacology*, 2013; 698(1-3): 31-38.
2. Benedec D., Vlase L., Oniga I., Toiu A., Tămaş M., Tipericiu B., Isoflavonoids From *Glycyrrhiza Sp.* And *Ononis Spinosa*. *Farmacia*, 2012; 60(5): 615-620.
3. Beyer J., Drummer O.H., Maurer H.H., Analysis of toxic alkaloids in body samples. *Forensic. Sci. Int.*, 2009; 185(1-3): 1-9.
4. Cascorbi I., Pharmacogenetics of cytochrome P450D6: genetic background and clinical implication. *Eur. J. Clin. Invest.*, 2003; 33(2): 17-22.
5. Daly J.W., Nicotinic agonists, antagonists, and modulators from natural sources. *Cell. Mol. Neurobiol.*, 2005; 25(3-4): 513-552.
6. Etter J.F., Cytisine for smoking cessation: a literature review and a meta-analysis. *Arch. Intern. Med.*, 2006; 166(15): 1553-1559.
7. Flores-Soto M.E., Bañuelos-Pineda J., Orozco-Suárez S., Schliebs R., Beas-Zárate C., Neuronal damage and changes in the expression of muscarinic acetylcholine receptor subtypes in the neonatal rat cerebral cortical upon exposure to sparteine, a quinolizidine alkaloid. *Int. J. Dev. Neurosci.*, 2006; 24(6): 401-410.
8. Hanganu D., Vlase L., Olah N., LC/MS Analysis Of Isoflavones From *Fabaceae Species* Extracts. *Farmacia*, 2010; 58(2): 177-183.
9. Ingelman-Sundberg M., Pharmacogenetics of cytochrome P450 and its applications in drug therapy: the past, present and future. *Trends Pharmacol. Sci.*, 2004; 25(4): 193-200.
10. Lourenco A.M., Maximo P., Ferreira L.M., Indolizidine and quinolizidine alkaloids structure and bioactivity, in *Bioactive Natural Products (part H)*. Elsevier Science BV, Amsterdam, 2002; vol 27: 233-298.
11. Tero-Vescan A., Imre S., Vari C.E., Oşan A., Dogaru M.T., Csödö C., Determination Of Some Isoflavonoids And Flavonoids From *Genista tinctoria L.* By HPLC-UV. *Farmacia*, 2009, Vol.LVII (1), 120-127.
12. Tutka P., Zatoński W., Cytisine for the treatment of nicotine addiction: from a molecule to therapeutic efficacy. *Pharmacol. Rep.*, 2005; 58: 777-798.
13. Vitale D.C., Piazza C., Melilli B., Drago F., Salomone S., Isoflavones: estrogenic activity, biological effect and bioavailability. *Eur. J. Drug Metab. Pharmacokinet.*, 2013; 38(1): 15-25.
14. *** Order no. 244/401/2005 of the Minister of Agriculture, Forestry and Rural Development and the Minister of Health of Romania.

Manuscript received: December 2013